Axial heterogeneity in basolateral AQP2 localization in rat kidney: effect of vasopressin

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TWO-THIRDS OF THE WATER entering the collecting duct is reabsorbed in the cortical collecting duct (CCD) due to osmotic equilibration with the near isosmotic cortical interstitium. The remaining urine enters the medullary collecting duct where it equilibrates with the hyperosmotic interstitium, resulting in excretion of a concentrated urine. The vasopressin-induced water permeability as well as the translocation of aquaporin-2 (AQP2) from intracellular vesicles to the apical plasma membrane in collecting duct principal cells is well characterized in the inner medullary collecting duct (IMCD) (17, 21, 26, 34, and for review, see Ref. 23), but virtually nothing is known about the trafficking of AQP2 in more proximal parts (17, 23). AQP2 is also found in the basolateral plasma membrane in IMCD principal cells (22), but it is uncertain whether vasopressin is involved in the basolateral targeting of AQP2. It is also unclear whether the same localization pattern of AQP2 is found in the proximal part of the collecting duct system, i.e., whether there is an axial heterogeneity in the basolateral expression of AQP2 along the connecting tubule (CNT) and collecting duct.

Recent studies have attempted to address the mechanisms underlying the basolateral targeting of AQP2. Vasopressin has been speculated to cause the basolateral targeting, since acute in vitro vasopressin treatment of kidney slices from normal rats has been shown to be associated with basolateral labeling of AQP2 (2). That oxytocin is also a potential candidate is shown by acute oxytocin treatment of normal rats, which induces distinct basolateral targeting of AQP2 in addition to apical targeting (10). In vitro studies performed by van Balkom et al. (33) have also indicated that hypertonicity may directly or indirectly induce targeting of AQP2 to the basolateral plasma membrane domains in AQP2-transfected Madin-Darby canine kidney (MDCK) cells. Finally, in some preparations, AQP2-transfected LLC-PK1 cells and primary cultured IMCD cells exhibited forskolin- or vasopressin-induced basolateral targeting of AQP2 (11, 16), whereas in other preparations MDCK cells and cells from a human cortical collecting duct cell line transfected with AQP2 showed only apical AQP2 targeting in response to vasopressin or forskolin stimulation (6, 31). Thus there is a great deal of confusion as to the presence or absence of regulated basolateral AQP2 targeting and whether this has any relevance in vivo.

Thus there is need for a rigorous study to define the subcellular localization (with a focus on the basolateral localization) of AQP2 along the CNT and collecting duct segments and, moreover, to establish whether there are major changes in the basolateral localization in conditions with altered vasopressin action. Specifically, in this study we investigated the basolateral localization of AQP2 along the CNT and the collecting
duct in normal rats and in vasopressin-deficient Brattleboro rats using immunocytochemistry at the light- and electron microscopic level. We furthermore investigated whether conditions with altered vasopres-
in action are associated with changes in AQP2 levels

Experimental Animals

Male Wistar rats were obtained from M & B (Ry, Denmark) and female homozygous Brattleboro rats from Harlan Netherlands (Horst, The Netherlands).

Experimental Protocols

Protocol 1. (1-[4-N-tert-butylcarbamoyl]-2-methoxybenzene sulfonyl)-5-ethoxy-3-spiro-[4-[2-morpholinoethoxy]cyclohexane indol-2-one phosphate monohydrate; cis-isomer (SR-121463B) is a nonpeptide, V₂-receptor antagonist (Sanofi-Synthélabo Recherche, Toulouse, France). SR-121463B was given via the femoral vein to normal rats briefl y anesthetized with halothane (n = 8). Control rats received saline (n = 8). Rats were placed in metabolic cages for 60 min, and anesthesia was then repeated. Kidneys were perfusion fixed for immunocytochemistry, paraffin embedding (eight controls and eight SR-121463B-treated rats), and cryosubstitution (four controls and four SR-121463B-treated rats).

Protocol 2. Brattleboro rats were treated subcutaneously with dDAVP (Sigma, Seelze, Germany; 500 ng in 500 µl saline/animal, n = 13). Control rats received subcutaneous saline (n = 11). Rats were placed in metabolic cages, and urine output and water intake were monitored. After 2 h of injection, anesthesia was repeated, and kidneys were perfusion fixed for immunocytochemistry, paraffin embedding (eight controls and nine dDAVP-treated rats), and cryosubstitution (eight controls and nine dDAVP-treated rats).

Protocol 3. For long-term treatment dDAVP, osmotic minipumps (Alzet, Palo Alto, CA) were filled with a solution of dDAVP and saline to give a dose of 250 ng/day (n = 6). Brattleboro rats were anesthetized, and the pump was placed subcutaneously. Controls were treated identically except that dDAVP was omitted from the solution (n = 5). Rats were put in metabolic cages with free access to food and water. Urine output and water intake were monitored for 6 days. After 6 days, rats were anesthetized, and the kidneys were perfusion fixed for immunocytochemistry.

Immunocytochemistry

Fixation. Kidneys were perfusion fixed with 1) 2% paraformaldehyde in 0.01 M NaOH, 0.075 M l-lysine, 0.0375 M Na₂HPO₄, pH 6.2; 2) 3% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4; or 3) 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4, via the abdominal aorta. Kidneys within the same experiment were identically fixed. Kidneys were postfixed for 30 min in 0.1 M sodium cacodylate. One kidney was subjected to paraffin embedding and one kidney for cryosubstitution.

Preparation of tissue for light and laser confocal microscopy. Before paraffin embedding, tissue blocks from whole kidneys were dehydrated in ethanol (2 h in 70, 96, and 99%, respectively) followed by incubation in xylene overnight. Paraffin sections (2 µm) were cut on a Leica RM 2126 microtome and dried overnight at 37°C. Sections were incubated with affinity-purified antibodies against rat or human AQP2, and labeling was visualized by use of peroxidase-conjugated sec-

RESULTS

Basolateral AQP2 Localization in Normal Rats

To investigate the basolateral distribution of AQP2 along the CNT and the collecting duct in normal rats, we performed immunocytochemistry at the light and electron microscopic level. Figure 1 shows the localization of AQP2 in the different kidney zones (cortex, outer and inner stripe of outer medulla (ISOM), and
inner medulla] in normal rats (Fig. 1, A–E, K, and L).

Immunoperoxidase microscopy revealed labeling of apical plasma membrane domains in all levels of the collecting duct in normal rats (Fig. 1, A–E). In the cortex, AQP2 was localized either in both apical and basolateral plasma membrane domains or mainly in apical plasma membrane domains (Fig. 1, A and B). These two strikingly different labeling patterns suggested a different localization of AQP2 in CNT and CCD. To distinguish between CNT and collecting ducts, we performed double immunolabeling for AQP2 and calbindin (Fig. 1, K–N). In the rat, calbindin is known to be abundantly expressed in intracellular domains and in the nucleus in cells of the DCT and CNT, where it is involved in calcium reabsorption. Calbindin is reported only to be present in a few collecting duct cells in the superficial cortex in the rat (1, 28). Thus we identified tubules staining positive for calbindin as CNT, whereas calbindin negative-staining tubules were identified as CCD. This double-labeling procedure revealed that AQP2 is localized in both apical and basolateral plasma membrane domains in CNT (Fig. 1K), whereas AQP2 is mainly situated in apical plasma membrane domains in CCD principal cells (Fig. 1L). Double immunoelectron microscopy using antibodies recognizing AQP2 and calbindin, respectively, confirmed this labeling pattern. In CNT cells, AQP2 labeling was present in both the apical and basolateral plasma membrane as well as intracellular vesicles and multivesicular bodies (Fig. 2, A and B). In CCD principal cells (calbindin negative), very little labeling was observed in the basal part of the cell and the basolateral plasma membrane (Fig. 3, A and B). AQP2 was present mainly in the apical part of the cell i.e., in the apical plasma membrane and in intracellular vesicles (Fig. 3, A and B). The differences in labeling of the basolateral plasma membrane in CNT and CCD were additionally confirmed by quantitation of the immunogold labeling in CNT cells and CCD principal cells (Table 1). The basolateral plasma membrane AQP2 labeling, determined as a fraction of total AQP2 cell labeling, was only 0.07 ± 0.02 (n = 4) in the CCD, whereas in the CNT the fraction was 0.33 ± 0.09 (n = 3). The total labeling density of AQP2 was lower in CNT compared with CCD (5.1 ± 1.7 vs. 23.8 ± 10.9 particles/μm²). Calbindin is reported to be present in the CNT, but the specific cell types expressing calbindin have not been reported. In the rat, the CNT consists of DCT cells, intercalated cells, CCT cells, and principal cells similar to those in the CCD (15). Therefore, we cannot conclude that all cells in the CNT containing both AQP2 and calbindin are true CNT cells and not principal cells present in the CNT. However, we observed calbindin/AQP2-positive cells that had the appearance of true CNT cells (intermediate between CCD principal cells and DCT cells), i.e., with deeper infoldings of the basolateral membrane compared with principal cells and with less mitochondria than the DCT cells (14). We also observed the presence of AQP2 in cells (both calbindin positive and negative) with an ultrastructure other than that of the CCT cells and the principal cells characterized by a lightly stained cytoplasm, i.e., staining with heavy metals in the uranyl acetate and lead citrate used for counterstaining at the electron microscopic level. These principal cells exhibited lower cell height and had a more darkly stained cytoplasm, and the infoldings of the basolateral membrane appeared less pronounced (Fig. 3A).

Immunoperoxidase microscopy revealed that AQP2 is mainly localized in apical plasma membrane domains in principal cells in the inner (IMCD) and outer stripe of the outer medullary collecting duct (OMCD) (Fig. 1, C and D). In IMCD principal cells, labeling was observed only in the apical plasma membrane. In the rat, calbindin is reported to be present in a few collecting duct cells in the superficial cortex in the rat (1, 28). Thus we identified tubules staining positive for calbindin as CNT, whereas calbindin negative-staining tubules were identified as CCD. This double-labeling procedure revealed that AQP2 is localized in both apical and basolateral plasma membrane domains in CNT and CCD (Fig. 1F–J). AQP2 localization was determined as a fraction of total labeling, decreased from 1.7 vs. 23.8 (n = 4 in each group). V_2-receptor antagonist treatment caused retrieval of AQP2 from apical plasma membrane domains to intracellular domains in all parts of the collecting duct including CCT, CNT, and OMCD as shown by light microscopy (Fig. 1, F–J, M, and N). Observations in IMCD are consistent with previous results showing retrieval of AQP2 from the apical plasma membrane in IMCD principal cells after 60 min of treatment with the V_2-receptor antagonist OPC-31260 (4). Labeling of basolateral plasma membrane domains was seen in the CNT and all parts of the collecting duct after SR-121463B treatment (Fig. 1, F–J, M, and N). Immunoelectron microscopy of ultrathin Lowicryl sections from the kidney cortex showed retrieval of AQP2 from the apical plasma membrane to intracellular vesicles present in the subapical part of the cell in CNT (Fig. 2C). There were no major changes in labeling of the basolateral plasma membrane in response to SR-121463B treatment (Fig. 2D). In CCD principal cells, SR-121463B treatment caused retrieval of AQP2 from the apical plasma membrane (Fig. 4). The apical plasma membrane labeling, determined as a fraction of total labeling, decreased from 0.20 ± 0.04 in controls (n = 4) to 0.006 ± 0.005 in SR-121463B-treated rats (n = 4; Table 1). In contrast, there was increased labeling of the basal part of the cell including the basolateral plasma membrane (Fig. 4). This was also further confirmed by quantitation of
immunogold labeling in the basolateral plasma membrane, which showed a major increase in the fraction of total labeling in the basolateral plasma membrane (0.29 ± 0.09 vs. 0.07 ± 0.02 in controls, \( n = 4 \)). The total labeling density was unchanged after SR-121463B treatment (21.0 ± 7.0 vs. 23.8 ± 10.9 in controls, \( n = 4 \)). In IMCD, there were no changes in the labeling density of the basolateral plasma membrane.
Basolateral AQP2 Localization in Kidneys from Brattleboro Rats

We investigated the subcellular localization of AQP2 in vasopressin-deficient Brattleboro rats by immunoperoxidase and immunofluorescent microscopy, in a fashion similar to the studies performed using normal rats (Figs. 6, A–D, and 7, A–E). The effect of short- and long-term dDAVP treatment will be described later (Figs. 6, E–H, and 7, F–O). Immunoperoxidase microscopy of sections of whole kidney revealed two different patterns of labeling in the cortex. In some segments, AQP2 was mainly localized apically (Fig. 6B), whereas in other segments AQP2 was mainly localized in the basolateral part of the cell (Fig. 6A). Double-labeling experiments with antibodies recognizing AQP2 and calbindin showed that cells of the CNT contained AQP2 labeling of basolateral plasma membrane domains (Fig. 6C), whereas CCD principal cells mainly contained apical AQP2 labeling (Fig. 6D). In general, the overall expression of AQP2 was lower in CNT compared with CCD. Double-labeling experiments at the immunoelectron microscopic level of ultrathin Lowicryl sections from the kidney cortex confirmed labeling of the basolateral plasma membrane in CNT cells (Figs. 8A and 10A). In CCD principal cells, little labeling was observed in the basal part of the cell including the basolateral plasma membrane (Figs. 9A and 10C). In the inner and outer stripe of the outer medulla, AQP2 was mainly localized in apical plasma membrane domains (Fig. 7, A and B), in IMCD principal cells, AQP2 was mainly localized in basolateral plasma membrane domains with the basolateral labeling being more intense in the inner third of IMCD (IM-3) compared with the outer third of IMCD (IM-1) (Fig. 7, C–E). Immunoelectron microscopy confirmed the subcellular distribution of AQP2 in sections from the outer third of IMCD. The labeling was mainly confined to the basolateral part of the cell, including the basolateral plasma membrane (Figs. 9C and 11A). Thus in untreated Brattleboro rats, AQP2 labeling of the basolateral plasma membrane is mainly observed in CNT cells and in IMCD principal cells, very similar to the labeling observed in normal rats.

Effects of 2-h dDAVP Treatment on Basolateral AQP2 Localization in Brattleboro Rats

Two hours of dDAVP treatment of Brattleboro rats caused a significant decrease in urine output (1.9 ± 0.4 vs. 20 ± 3.3 ml/h, n = 13). Immunocytochemistry showed that short-term dDAVP treatment induces a marked increase in labeling of apical plasma membrane domains in cells of the CNT (Fig. 6E) and in IMCD principal cells (Fig. 7, H–J). In contrast, in IMCD principal cells, AQP2 labeling of the basolateral plasma membrane domains was decreased after short-term dDAVP treatment (Fig. 7, H–J). Immunoelectron microscopy showed no significant changes in the labeling of the basolateral plasma membrane in CNT cells and in CCD principal cells (Figs. 8B and 9B), whereas in the IMCD the AQP2 immunogold labeling density of the basolateral plasma membrane was slightly decreased (Fig. 9D). Thus after 2 h of acute dDAVP treatment there is no increase in the basolateral plasma membrane expression of AQP2.

Effects of 6-Day dDAVP Treatment on Basolateral AQP2 Localization in Brattleboro Rats

Six days of dDAVP treatment caused a dramatic decrease in urine output (12 ± 1.4 vs. 219 ± 20 ml/24 h, n = 9, the last 24 h of treatment). Immunocytochemistry showed that long-term dDAVP treatment of Brattleboro rats induced an increase in apical plasma membrane domain labeling in all parts of the collecting duct (Figs. 6, G and H, and 7, K–O). Labeling of basolateral plasma membrane domains was maintained in CNT cells (Fig. 6G) and increased in CCD and IMCD.
OSOM principal cells (Figs. 6H and 7K). The basolateral plasma membrane domain labeling was decreased in IMCD principal cells (Fig. 7, M–O). Immunoelectron microscopy of sections of kidney cortex showed that the labeling of the basolateral plasma membrane was moderately increased in CNT cells (Fig. 10B). There was a major increase in labeling of the basal part of the cell, including the basolateral plasma membrane in CCD

Fig. 2. Electron micrographs of ultrathin HM20 Lowicryl sections from kidney cortex (CNT) of normal rats (A and B) and rats treated with SR-121463B for 60 min (C and D). Sections were double immunogold labeled for AQP2 (large arrows, large gold particles) and calbindin (small arrows, small gold particles) to distinguish between CNT and CCD. The presence of calbindin as well as the cell ultrastructure indicates that the cells are CNT cells. A: in the apical part of CNT cells in normal rats AQP2 labeling is associated with the apical plasma membrane (large arrows), intracellular vesicles (arrowheads), and multivesicular bodies (MVB). B: AQP2 labeling is also seen in the basal part of the cells including the BLM. C: SR-121463B treatment causes retrieval of AQP2 from the apical plasma membrane to intracellular vesicles (arrowheads) present in the subapical part of the cell. D: there are no major changes in the labeling of the BLM after SR-121463B treatment (large arrows). L, lumen; M, mitochondrion. Magnification: ×63,000 in A; ×46,500 in B–D.
principal cells (Fig. 10D). Quantitation of immunogold labeling showed that the labeling of the basolateral plasma membrane taken as a fraction of total cell labeling was increased in dDAVP-treated rats (0.21 ± 0.04 vs. 0.05 ± 0.01 in controls, n = 4). The total labeling density in CCD was unchanged (8.8 ± 1.4 vs. 8.1 ± 1.4 in controls, n = 4). After long-term dDAVP treatment, AQP2/calbindin-positive tubules were seen with a higher frequency compared with control Brattleboro rats. Some of these cells had the appearance of CCD principal cells, suggesting that part of the CNT becomes calbindin positive after dDAVP exposure or that there may also be a longer region of the CNT that expresses AQP2 in response to long-term treatment with dDAVP. Consistent with this, previous studies have indicated that the CNT of Brattleboro rats has an upstream region lacking AQP2 and that chronic vasoressin treatment induces AQP2 expression in this region (5). In the outer third of the IMCD, AQP2 labeling in the basal part of the cells was significantly decreased in response to long-term dDAVP treatment, whereas the labeling density of the basolateral plasma membrane was slightly increased (Fig. 11B). The overall expression of AQP2 also increased in IMCD. Thus long-term dDAVP treatment causes a major increase in the basolateral plasma membrane expression of AQP2 in CCD principal cells, which was not associated with increased protein expression in CCD (i.e., determined as total AQP2 immunogold labeling). In contrast, CNT cells and IMCD principal cells exhibit only

Fig. 3. Electron micrographs of ultrathin HM20 Lowicryl sections from kidney cortex (CCD) of normal rats (A and B). To distinguish between CNT and CCD, we double-immunogold labeled sections for AQP2 and calbindin. The absence of calbindin shows that the cells are CCD principal cells. AQP2 is mainly present in the apical part of the cell in the apical plasma membrane (arrows) and in intracellular vesicles (arrowheads). Little labeling is present in the basal part of the cell including the BLM (arrows). Besides principal cells with light staining of cytoplasm, i.e., staining with heavy metals in the uranyl acetate and lead citrate used for counterstaining at the electron microscopic level (B), we also observed principal cells that exhibited lower cell height and had a more darkly stained cytoplasm and less pronounced infoldings of the basal membrane (A). PC, principal cell; IC, intercalated cell. Magnification: ×46.500 in A, ×34.500 in B.
a moderate increase in basolateral plasma membrane labeling.

**DISCUSSION**

Immunocytochemistry at the light- and electron microscopical level revealed an axial heterogeneity in the basolateral localization of AQP2 along the CNT and the collecting duct segments in normal rats and in vasopressin-deficient Brattleboro rats. AQP2 labeling of the basolateral part of the cell, including the basolateral plasma membrane, was seen mainly in CNT cells and IMCD principal cells, whereas CCD and OMCD principal cells exhibited little basolateral labeling. Short-term dDAVP treatment of Brattleboro rats caused no net increase in the labeling of the basolateral plasma membrane in any segment.

Table 1. Quantitation of immunogold labeling of AQP2 in CCD and CNT

<table>
<thead>
<tr>
<th>Condition</th>
<th>BLM (Fraction of cell total labeling)</th>
<th>APM (Fraction of cell total labeling)</th>
<th>Intracellular (Fraction of cell total labeling)</th>
<th>Area, ( \mu m^2 )</th>
<th>Total Labeling Density, Particles/( \mu m^2 )</th>
<th>( n )</th>
</tr>
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<tbody>
<tr>
<td>Control, CNT</td>
<td>0.33 ± 0.09</td>
<td>0.12 ± 0.01</td>
<td>0.55 ± 0.08</td>
<td>117</td>
<td>5.1 ± 1.7</td>
<td>3</td>
</tr>
<tr>
<td>Control, CCD</td>
<td>0.07 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.71 ± 0.05</td>
<td>72</td>
<td>23.8 ± 10.9</td>
<td>4</td>
</tr>
<tr>
<td>SR-121463B, CCD</td>
<td>0.29 ± 0.09</td>
<td>0.006 ± 0.005</td>
<td>0.71 ± 0.09</td>
<td>90</td>
<td>21.0 ± 7.0</td>
<td>4</td>
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Values are means ± SE. Gold particles were counted in connecting tubules (CNT) and cortical collecting ducts (CCD) of control rats and in CCD of SR-121463B-treated rats. Values are expressed as fraction of total cell labeling of the basolateral plasma membrane (BLM), apical plasma membrane (APM), and of the intracellular labeling. Area, total area counted; \( n \), number of animals.

Fig. 4. Electron micrographs of ultra-thin HM20 Lowicryl sections from kidney cortex (CCD) of rats treated with SR-121463B for 60 min. Treatment with SR-121463B causes retrieval of AQP2 from the apical plasma membrane to intracellular vesicles (arrowheads) and the BLM (arrows). M, mitochondrion. Magnification, \( \times 34,500 \).
whereas long-term (6 days) dDAVP treatment of Brattleboro rats caused a major increase in basolateral plasma membrane AQP2 labeling in CCD principal cells, but not in IMCD cells. Acute V2-receptor antagonist treatment of normal rats caused no decrease in basolateral plasma membrane AQP2 labeling. In contrast, increased AQP2 expression in the basolateral plasma membrane was observed in CCD in response to acute V2-receptor antagonist treatment.

Axial Heterogeneity in Basolateral Localization of AQP2

Our results demonstrated that there is an axial heterogeneity in the basolateral AQP2 localization. AQP2 labeling of the basolateral plasma membrane was most prominent in CNT cells and in IMCD principal cells in both normal rats and Brattleboro rats. AQP2 labeling of the basolateral plasma membrane has been reported previously in IMCD principal cells in normal rats, as well as in a undefined cortical segment i.e., collecting duct or CNT (22). Coleman et al. (5) have recently reported that AQP2 occurs in basolateral plasma membrane domains of CNT cells to a variable extent, and Jeon et al. (10) have also shown labeling of basolateral plasma membrane domains in CNT cells. In this study, we provide evidence of extensive AQP2 labeling in the basolateral plasma membrane using quantitative immunoelectron microscopy.

Fig. 5. Electron micrographs of ultrathin HM20 Lowicryl sections from kidney inner medulla of a normal rat (A) and a rat treated with SR-121463B for 60 min (B). Sections were immunogold labeled for AQP2. A: AQP2 is present in the BLM in normal rats (arrows). B: SR-121463B treatment causes no changes in AQP2 labeling of the BLM. Magnification, ×34,500.
The mechanisms involved in the targeting of AQP2 expression to the basolateral plasma membrane are not well defined. Van Balkom et al. (33) tested the hypothesis that the presence of AQP2 at the basolateral plasma membrane may be due to heterotetramerization with AQP3 and AQP4. However, coexpression studies in oocytes indicated that this is not the case. Hypertonicity appears to have an effect on the expression level of some renal aquaporins. A hypertonicity-responsive element has been identified in the human AQP1 and AQP2 genes (18, 30), and the expression of AQP3 has been shown to increase in response to hypertonicity in MDCK cells (19). Recent in vitro studies by van Balkom et al. (33) have suggested that hypertonicity is involved in basolateral targeting of AQP2. Using different osmolytes, they showed that a gradual increase (over a 3-day period) in osmolarity of the surrounding medium causes basolateral AQP2 targeting in response to forskolin stimulation in AQP2-transfected MDCK cells. This theoretically could explain the basolateral expression pattern in IMCD in normal rats being in a hypertonic milieu (exposed to high basolateral tonicity). But this probably does not offer an explanation for the basolateral localization of AQP2 in CNT, nor the difference in basolateral AQP2 expression in CNT and CCD. In contrast to the CNT, we observed little or no AQP2 in the basolateral plasma membrane in CCD principal cells. Although the absence of basolateral AQP2 in CCD principal cells correlates with the hypothesis of an important role of hypertonicity on basolateral AQP2 targeting, the extensive basolateral labeling in CNT contradicts this hypothesis. The CNT and CCD are both embedded in one to plasma isotonic interstitium, and the luminal osmolality is low, ~100 mosmol/kgH2O. Also in the kidney medulla there are differences in basolateral membrane expression of AQP2. In the medullary collecting duct, which is embedded in a hyperosmotic interstitium (the luminal osmolality ranging from 200 to 3,000 mosmol/kgH2O in mammals), we observed significant AQP2 labeling of the basolateral plasma membrane in the IMCD. In Brattleboro rats the basolateral expression was more intense in the inner third of IMCD. In contrast, there was no significant basolateral AQP2 labeling in collecting duct principal cells in ISOM. Thus there is a progressive decrease in basolateral AQP2 expression going from the initial IMCD to the OMCD with a change in the presence or absence of AQP2 in the basolateral plasma membrane between the initial IMCD and the terminal ISOM collecting duct. Although the interstitial osmolality also de-
creases through the medulla, there is no evidence for a similar major change in the interstitial osmolality in the same region, which could explain the significant differences in basolateral AQP2 expression. Thus the hypertonicity theory most likely does not explain the basolateral labeling in the CNT in normal rats nor the basolateral AQP2 labeling in Brattleboro rat CNT and IMCD principal cells, which are in a more or less permanent state of water diuresis. Moreover, preliminary results revealed a maintained or even increased basolateral AQP2 labeling in IMCD principal cells in kidneys from Wistar rats treated with the loop diuretic
furosemide in vivo (Christensen and Nielsen, unpublished observations). This also speaks against the hypothesis that hypertonicity induces basolateral AQP2 targeting in rat kidney.

The presence of basolateral plasma membrane labeling in vasopressin-deficient Brattleboro rats indicate that factors other than vasopressin are responsible for the expression of AQP2 at the basolateral plasma membrane. There may, however, be an extrahypothalamic production of vasopressin or even a low hypothalamic production of vasopressin in Brattleboro rats (32), and it could be speculated that even though these potentially very low vasopressin levels have no physiological effects (i.e., antiuretic effect), the levels may be high enough to cause basolateral AQP2 targeting. This would be consistent with previous findings that the overall expression of AQP2 in Brattleboro rats decreases in response to V2-receptor antagonist treatment (25). However, if low vasopressin levels are responsible for the basolateral plasma membrane labeling in untreated Brattleboro rats, it would expected that acute vasopressin treatment would increase basolateral plasma membrane labeling, which is not the case. Oxytocin is another hormone with antiuretic effect (3), and treatment of normal rats with this hormone has been reported to induce both apical and basolateral targeting of AQP2, an effect that was abolished by pretreatment with a V2-receptor antagonist (10). Thus oxytocin may be involved in basolateral AQP2 targeting in Brattleboro rats.

Fig. 8. Electron micrographs of ultrathin HM20 Lowicryl sections from the kidney cortex (CNT) of an untreated Brattleboro rat (A) and a Brattleboro rat treated with dDAVP for 2 h (B). Sections were double immunogold labeled for AQP2 (large arrows, large gold particles) and calbindin (small arrows, small gold particles) to distinguish between CNT and CCD. The presence of calbindin as well as the cell ultrastructure indicates that the cells are CNT cells. A: AQP2 is present in the basal part of the cell including the BLM in an untreated Brattleboro rat (large arrows). B: 2 h after dDAVP injection there is no major changes in AQP2 labeling of the BLM (large arrows). Magnification, ×34,500.
Acute V2-Receptor Agonist Treatment Causes No Increase in Basolateral AQP2 Labeling

Our data show no evidence of basolateral targeting of AQP2 in response to 2-h dDAVP treatment in Brattleboro rats. This was the case in all AQP2-containing segments in the kidney tubule. Recent nonphysiological in vitro studies have shown that stimulation of dissected kidney slices (from normal rats) with vasopressin and forskolin for 15 min causes translocation of AQP2 from cytoplasmic vesicles to basolateral plasma membrane domains in IMCD principal cells, with the most prominent basolateral staining in the distal part of IMCD (33). However, when the experiment was performed with kidney slices from Brattleboro rats, there was mainly staining of apical plasma membrane domains after 15 min of forskolin/vasopressin treatment, consistent with our results (33). It could be argued that treatment of normal rats with dDAVP for a shorter period than 2 h, e.g., minutes, would cause basolateral targeting of AQP2 in vivo. However, previous studies have indicated that this is not the case. Treatment of normal rats with dDAVP for 20 min caused no increase in the labeling density of the basolateral plasma membrane in IMCD principal cells (17), and there was no change in the labeling of the basolateral plasma membrane in IMCD principal cells of Brattleboro rats after 15 min of vasopressin stimulation (34). Furthermore, it has recently been shown that, after 30 min of vasopressin treatment, there is no increase in AQP2 labeling of basolateral plasma membrane domains in any regions of the kidney (10).

Long-Term V2-receptor Agonist Treatment Increases Basolateral AQP2 Labeling

AQP2 labeling of the basolateral plasma membrane was only prominent in IMCD principal cells and CNT cells in untreated Brattleboro rats, and long-term dDAVP treatment caused only minor increases in basolateral plasma membrane AQP2 labeling. In contrast, little AQP2 labeling of the basolateral plasma membrane was observed in CCD principal cells in untreated Brattleboro rats, and in this segment a major increase in AQP2 expression in the basolateral plasma membrane was observed after long-term dDAVP treatment. Quantitation of gold particles in CCD principal cells showed a significantly increased AQP2 labeling in the basolateral plasma membrane (expressed as a fraction of total cell labeling) after long-term dDAVP treatment. Thus this demonstrates that the increase in basolateral AQP2 labeling in the

![Fig. 9. Electron micrographs of ultrathin HM20 Lowicryl sections from kidney cortex (CCD, A and B) and inner medulla (C and D) of untreated Brattleboro rats (A and C) and Brattleboro rats treated with dDAVP for 2 h (B and D). A and B: sections from cortex were double-immunogold labeled for AQP2 and calbindin to distinguish between CNT and CCD. The absence of calbindin shows that the cells are CNT principal cells. A: in CCD of an untreated Brattleboro rat little labeling of the BLM is seen (arrows). B: 2 h after dDAVP injection there is no major change in AQP2 labeling of the BLM (arrows). C: in the inner medulla, AQP2 is present in the basal part of the cell including the BLM in an untreated Brattleboro rat (arrows). D: 2 h after dDAVP injection, there is a slight decrease in AQP2 labeling of the BLM (arrows). Magnification, ×34,500.](http://ajprenal.physiology.org/FIG9.jpg)
CCD was not a consequence of increased AQP2 protein expression as previously demonstrated (7, 29). Increased AQP3 protein expression has been shown in response to long-term dDAVP treatment of Brattleboro rats (29). AQP3 is present mainly in the basolateral plasma membrane, and AQP3 trafficking is not regulated by acute vasopressin (8). Only a small fraction of AQP3 is located in intracellular vesicles, most likely representing newly synthesized AQP3 protein in transit from the Golgi to the basolateral plasma membrane (8). Thus similar to AQP3, AQP2 may be constitutively expressed at the basolateral plasma membrane and participate in water transport across the basolateral membrane, although the contribution of AQP2 as an exit pathway is likely quite small, since AQP3 knockout mice are severely polyuric (13). However, the fact that AQP3 knockout mice do survive in contrast to AQP2 and vasopressin V2-receptor knockout mice may

Fig. 10. Electron micrographs of ultrathin HM20 Lowicryl sections from the kidney cortex of untreated Brattleboro rats (A and C) and Brattleboro rats treated with dDAVP for 6 days (B and D). Sections were double-immunogold labeled for AQP2 (large arrows, large gold particles) and calbindin (small arrows, small gold particles) to distinguish between CNT and CCD. The presence of calbindin as well as the cell ultrastructure indicates that the cells are CNT cells (A and B). A: in CNT of an untreated Brattleboro rat AQP2 is present in the BLM (large arrows). B: AQP2 labeling of the BLM is moderately increased after 6 days’ dDAVP treatment in CNT cells (large arrows). C: in CCD of an untreated Brattleboro rat little labeling of the BLM is seen (large arrows). D: 6 days of dDAVP treatment causes a major increase in AQP2 labeling of the basal part of the cell including the BLM in CCD principal cells (large arrows). The absence of calbindin shows that the cells are CCD principal cells. Magnification, ×34,500.
be related to a role of AQP4 and, potentially, also a role of AQP2 in the basolateral plasma membranes.

Changes in the Subcellular Localization of AQP2 in Response to Acute V2-Receptor Antagonist Treatment

Immunoelectron microscopy revealed that V2-receptor antagonist treatment causes increased labeling of AQP2 in the basolateral plasma membrane of CCD principal cells, whereas the basolateral labeling density is unchanged in CNT and IMCD. Interestingly, the effect of V2-receptor antagonist treatment on the subcellular localization of AQP2 differed considerably in CCD and CNT. Although AQP2 was internalized from the apical plasma membrane in both CNT and CCD, AQP2 was retrieved to intracellular vesicles in the subapical part of the CNT cell with unchanged labeling of the basolateral plasma membrane, whereas in the CCD AQP2 was retrieved to the basal part of the cell with increased labeling of the basolateral plasma membrane. The fact that we did not observe a retrieval of AQP2 from the basolateral plasma membrane strongly supports the view that AQP2 is not targeted to the basolateral plasma membrane by short-term V2-receptor stimulation (vasopressin or dDAVP).

Basolateral Localization of AQP2

The results demonstrate that there is a significant basolateral AQP2 targeting in connecting tubule cells and in IMCD cells. There is no change in basolateral targeting in response to acute vasopressin treatment of vasopressin-deficient Brattleboro rats, consistent with previous studies (10, 17, 34). This indicates that basolateral targeting is not vasopressin mediated, which is consistent with the observation that significant basolateral AQP2 targeting is selectively seen in the CNT and IMCD but not or to a much lesser extent in the CCD and OMCD. This selective basolateral targeting also strongly speaks against an effect of interstitial osmolality, since it is unlikely that there are major differences in interstitial osmolality associated with the CNT and CCD. Thus the signaling mechanisms remain uncertain, but the axial heterogeneity strongly suggests that cell-specific mechanisms are involved. It should be emphasized that there, indeed, are marked ultrastructural differences between, e.g., CNT cells, collecting duct principal cells in the CCD, and IMCD cells. Moreover, there are marked differences in the expression pattern of transporters, e.g., the vasopressin-regulated urea transporter, which is expressed only in the terminal IMCD, not in principals cells in more proximal parts of the collecting duct (for a review, see Ref. 27). Also, AQP4 is expressed to a greater extent in the IMCD than in CCD. Thus cell-specific mechanisms are likely to be involved in basolateral AQP2 expression. The targeting mechanisms are most likely specific to AQP2 since epithelial Na\(^+\) channel subunits are expressed only in the apical plasma membrane and in vesicles but not in the basolateral plasma.

Fig. 11. Electron micrographs of ultrathin HM20 Lowicryl sections from kidney inner medulla of an untreated Brattleboro rat (A) and a Brattleboro rat treated with dDAVP for 6 days (B). A: AQP2 is present in the basal part of the cell including the BLM in an untreated Brattleboro rat (arrows). B: there is a slight increase in AQP2 labeling of the BLM after 6 days of dDAVP treatment (arrows). MVB, multivesicular body. Magnification, ×34,500.
membrane (9, 12). Additional studies are necessary to establish the signaling and potential regulation of basolateral AQP2.

Summary

Our results show that there is a marked axial heterogeneity in the expression levels of AQP2 in the basolateral plasma membrane along the CNT and collecting duct subsegments. AQP2 expression in the basolateral plasma membrane was not increased by short-term dDAVP treatment in Brattleboro rats. Moreover, acute V$_2$-receptor antagonist treatment of normal rats did not cause a decrease in basolateral plasma membrane labeling. Long-term (6 days) dDAVP treatment of Brattleboro rats caused major increases in basolateral plasma membrane labeling of AQP2 in CCD principal cells and moderate increases in CNT cells and IMCD principal cells.

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